# *NFE2L2* Gene Mutation in Male Japanese Squamous Cell Carcinoma of the Lung

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**Introduction:** Recently, the nuclear factor (erythroid derived 2)-like 2 (*NFE2L2*) gene mutations were identified in lung cancer. The constitutive activation of *NFE2L2* in lung cancer cells promotes tumorigenicity. However, the correlation between *NFE2L2* mutation status and clinicopathologic features of lung cancer has not been well characterized.

**Methods:** We have investigated *NFE2L2* gene mutation status in 263 surgically treated lung cancer cases at Nagoya City University Hospital. The *NFE2L2* mutation was analyzed by direct sequencing of cDNA.

**Results:** We detected 13 cases (5.1%) of *NFE2L2* mutation in our cohort; all were male and all had a squamous histology. *EGFR* mutations were present in 78 patients (30.8%). The *NFE2L2* mutation was exclusive with *EGFR* mutations. The *NFE2L2* mutation tended to be more frequently found in patients with advanced stages. The patients with *NFE2L2* mutation ($n = 13$, 8 were dead) had significantly worse prognosis than the patient with wild type *NFE2L2* ($n = 250$, 72 were dead) (Log-rank test, $p = 0.0032$, Breslow-Gehan-Wilcoxon test, $p = 0.0028$).

**Conclusion:** *NFE2L2* mutations might play a role in tumor prognosis of squamous cell carcinoma of the lung.

**Key Words:** NFE2L2, Squamous cell carcinoma, EGFR, Tumor progression, mutation.

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Lung cancer is a major cause of death from malignant diseases, due to its high incidence, malignant behavior and lack of major advancements in treatment strategy.[1] Lung cancer was the leading indication for respiratory surgery (42.2%) in 1998 in Japan.[2] More than 15,000 patients underwent surgical operation at Japanese institutions in 1998.[2]

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The nuclear factor (erythroid derived 2)- like 2 (NFE2L2) is a master transcriptional activator of genes encoding many cytoprotective enzymes that are induced in response to environmental and endogenously derived oxidative/electrophilic agents.[3–5] In normal cells, NFE2L2 is a cap 'n' collar basic leucine zipper transcription factor. *NFE2L2*-deficient mice are highly susceptible to chemically induced carcinogenesis of multiple organs.[6,7] A previous report showed that RNAi-mediated silencing of *NFE2L2* gene expression in non-small cell lung cancer inhibited tumor growth.[8] *NFE2L2* gene promoter polymorphism has been identified and was suggested to be correlated with carcinogenesis.[9] More recently, *NFE2L2* mutation was identified in lung cancer tissue and cell lines.[10] Somatic mutations occurred in the coding region of *NFE2L2* mutations were found frequently among patients with a history of smoking or suffering from squamous cell carcinoma and correlated with poor prognosis.[10]

We investigated *NFE2L2* mutation status an *N*-terminal domain by direct sequencing in Japanese lung cancer. The findings were analyzed in reference to the clinicopathologic features of the lung cancer.

## PATIENTS AND METHODS

### Patients

The study group included 263 patients with lung cancer who had undergone surgery at the Department of Surgery II, Nagoya City University Medical School between 1997 and 2006. The lung tumors were classified according to the general rule for clinical and pathologic record of lung cancer in Japan. All tumor samples were immediately frozen and stored at −80°C until assayed.

The clinical and pathologic characteristics of the 263 patients with lung cancer were as follows: 150 cases (57.0%) at stage I, 46 at stage II (17.5%), 63 (24.0%) at stage IIII, and four at stage IV. The mean age was 64.8 years (range, 38–83). Among the 263 patients with lung cancer, 190 (72.2%) were male and 73 (27.8%) were nonsmokers. The samples from these patients had been already sequenced for *EGFR* before.[11–14]

### PCR Assays for NFE2L2

Total RNA was extracted from lung cancer tissues using Isogen kit (Nippon gene, Tokyo, Japan) according to

the manufacturers' instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 200 ng/ml. About 10 cases were excluded for each assay because tumor cells were too few to sufficiently extract tumor RNA. RNA (1 $\mu$g) was reverse transcribed by Superscript II enzyme (Gibco BRL, Gaithersburg, MD) with 0.5 $\mu$g oligo $(dT)_{12-16}$ (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The reaction mixture was incubated at 42°C for 50 minutes and then at 72°C for 15 minutes. We then used 1 $\mu$L of each DNA for polymerase chain reaction (PCR) analyses. The PCR reactions were performed using LA-*Taq* kit (Takara Bio Inc, Shiga, Japan) in a 50-$\mu$L reaction volume. The primer sequences for *NFE2L2* gene were as follows: the forward primer, 5-TCATGATGGACTTGGAGCTG-3 and the reverse primer, 5-CTACAAACGGGAATGTCTGC-3. The cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 40 cycles at 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 30 seconds. The products were purified by Qiagen PCR purification kit (Qiagen, Valencia, CA). Amplified DNAs were separated on 1% agarose gels, and the bands were visualized by ethidium bromide and photographed under ultraviolet transillumination. These samples were sequenced by ABI prism 3100 analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan) and analyzed by BLAST and chromatograms by manual review.

## Statistical Analysis

Statistical analyses were done using the Mann-Whitney *U* test for unpaired samples and Wilcoxon's signed rank test for paired samples. Linear relationships between variables were determined by means of simple linear regression. Correlation coefficients were determined by rank correlation using Spearman's test and $\chi^2$ test. The overall survival of patients with lung cancer was examined by the Kaplan-Meier methods, and differences were examined by the Log-rank test. All analysis was done using the Stat-View software package (Abacus Concepts Inc. Berkeley, CA) and was considered significant when the *p* value was less than 0.05.

# RESULTS

## *NFE2L2* Gene Mutation Status in Japanese Patients with Lung Cancer

We used cDNA from the lung cancer tissue to directly sequence the *NFE2L2* gene after PCR reaction, 13 of 263 patients had mutation in the *NFE2L2* gene (Figure 1). Six were known mutations (V32G, R34Q, E79K, and E79Q) and seven were novel mutations (D29Y, R34P, R34G, D77G, and E79D) (Table 1). The *NFE2L2* mutations were clustered in exon 2 and resulted in amino acid changes in either the DLG or the ETGE motif of the regulatory Neh2 domain.[15] Matched adjacent normal lung tissues were available in 10; in all of them, the sequence was wild type, suggesting that the mutations were somatic. All 13 patients were male and 12 were smokers. Four (30.8%) were stage I, four were stage II, and five (38.5%) were stage III. In addition, *NFE2L2* mutation had a tendency to be more frequently found in advanced stages (pStage II-IV versus I; $p = 0.0812$). Seventy-eight patients had *EGFR* mutation at the kinase domain. The

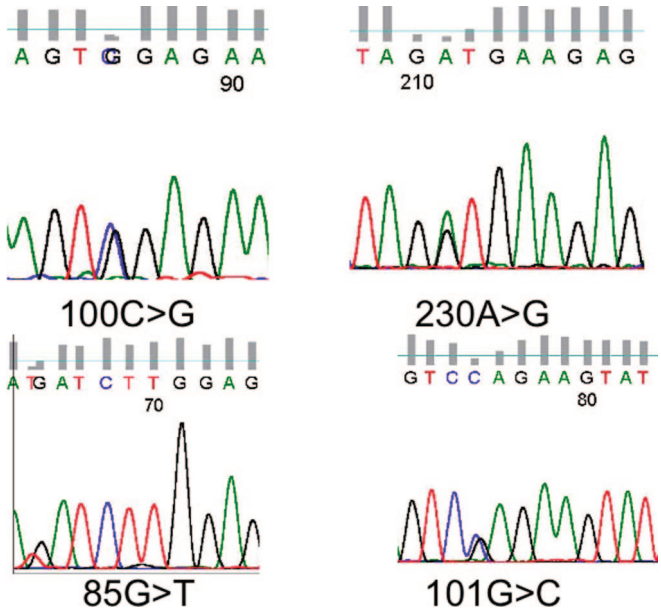


**FIGURE 1.** Five novel *NFE2L2* gene mutations. GAT > TAT (D29Y, Asp > Tyr), CGG > CCA (R34P, Arg > Pro), CGA > GGA (R34G, Arg > Gly), GAT > GGT (D77G, Asp > Gly), and GAG > GAC (E79D, Glu > Asp).

**TABLE 1.** NFE2L2 Mutations

| Sample | Nucleotide Mutation | Amino Acid Change | Histology | Gender | Age |
|---|---|---|---|---|---|
| 06–02 | 85 G-T | D29Y (Asp > Tyr) | SCC | Male | 58 |
| 98–14 | 95 T-G | V32G (Val > Gly) | SCC | Male | 74 |
| 98–48 | 100 C-G | R34G (Arg > Gly) | SCC | Male | 74 |
| 98–13 | 101 G-C | R34P (Arg > Pro) | SCC | Male | 63 |
| 01–18 | 101 G-A | R34Q (Arg > Gln) | SCC | Male | 65 |
| 02–39 | 101 G-A | R34Q (Arg > Gln) | SCC | Male | 66 |
| 03–40 | 101 G-A | R34Q (Arg > Gln) | SCC | Male | 79 |
| 01–25 | 230 A-G | D77G (Asp > Gly) | SCC | Male | 76 |
| 02–25 | 235 G-C | E79Q (Glu > Gln) | SCC | Male | 77 |
| 06–42 | 235 G-A | E79K (Glu > Lys) | SCC | Male | 77 |

*NFE2L2* mutation was exclusive with the *EGFR* mutation. Thirty-four patients including one *NFE2L2* mutant patient received adjuvant or neoadjuvant chemotherapy.

The overall survival of 263 patients with lung cancer with follow-up through December 31, 2009, was studied in reference to the *NFE2L2* mutation status. The patients with *NFE2L2* mutation ($n = 13$, 8 were dead) had significantly worse prognosis than the patients with wild type *NFE2L2* ($n = 250$, 72 were dead) (Log-rank test, $p = 0.0032$, Breslow-Gehan-Wilcoxon test; $p = 0.0028$) (Figure 2). The multivariate analyses revealed that pathologic stage ($p < 0.0001$, hazard ratio = 2.989, 1.864–4.793) and *NFE2L2* mutation ($p = 0.0379$, hazard ratio = 2.183, 1.045–4.566) were the significant prognostic factors (Table 2). Within the patients with squamous cell carcinoma, the patients with *NFE2L2* mutation ($n = 13$, 8 were dead) had significantly worse prognosis than the patients with wild type *NFE2L2*

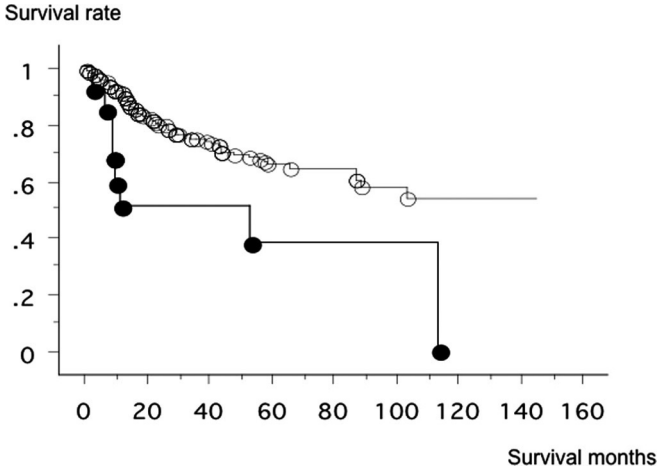


**FIGURE 2.** The patient with *NFE2L2* mutation in coding region ($n$ = 13, 8 were dead; mean survival = 54.7 months) was significantly worse prognosis than the patient with wild type *NFE2L2* ($n$ = 250, 72 were dead; mean survival = 73.4 months) (Log-rank test, $p$ = 0.0032, Breslow-Gehan-Wilcoxon test, $p$ = 0.0028).

**TABLE 2.** Clinicopathological Data of 263 Patients with Lung Cancer

| | *NFE2L2* Gene Status | | |
|---|---|---|---|
| **Factors** | **Wild-Type Patients** | **Mutant Patients** | ***p*** |
| Mean age (yr) | | | |
| 64.9 ± 9.0 | 250 | 13 | |
| Stage | | | |
| I | 146 (58.4%) | 4 (30.8%) | 0.0812 |
| II-IV | 104 (41.6%) | 9 (69.2%) | |
| Lymph node metastasis | | | |
| N0 | 177 (70.8%) | 6 (46.2%) | 0.0697 |
| N+ | 73 (29.2%) | 7 (53.8%) | |
| Smoking | | | |
| Never smoker | 76 (30.4%) | 1 (7.7%) | 0.1165 |
| Smoker | 174 (69.6%) | 12 (92.3%) | |
| EGFR mutation | | | |
| Wild type | 142 (56.8%) | 13 (100%) | 0.1207 |
| Mutation | 78 (43.2%) | 0 (0%) | |
| Pathological subtypes | | | |
| SCC | 109 (43.6%) | 13 (100%) | 0.0001 |
| Non-SCC | 141 (56.4%) | 0 (0%) | |
| Age | | | |
| ≦65 | 112 (44.8%) | 5 (38.5%) | 0.7784 |
| >65 | 138 (55.2%) | 8 (61.5%) | |
| Gender | | | |
| Male | 177 (69.2%) | 13 (100%) | 0.0001 |
| Female | 73 (30.8%) | 0 (0%) | |

N+, lymph node metastasis positive; SCC, squamous cell carcinoma.

($n$ = 111, 32 were dead) (Log-rank test, $p$ = 0.0127, Breslow-Gehan-Wilcoxon test; $p$ = 0.0212). Within the male patients with lung cancer, the patients with *NFE2L2* mutation ($n$ = 13, 8 were dead) had significantly worse prognosis than the patients with wild type *NFE2L2* ($n$ = 177, 57 were dead) (Log-rank test, $p$ = 0.0193, Breslow-Gehan-Wilcoxon test; $p$ = 0.018) (Figure 3).

## DISCUSSION

We sequenced the *NFE2L2* gene using the cDNA from 263 lung cancer tissue and found that *NFE2L2* mutation was correlated with male gender, smoking history, squamous histology, and poor prognosis. This was in agreement with a previous report and suggests a relationship between the *NFE2L2* mutation and tobacco smoke.[10] The possible constitutive activation of *NFE2L2* by somatic mutation at E3 ligase recognition sites of the Neh2 domain has been identified in lung cancers and head and neck cancers.[10]

The preferential *NEF2L2* mutations among patients diagnosed with squamous cell carcinoma could also be explained by stress-related *NFE2L2*-dependent intervention to squamous cell lineage differentiation. Malignant development induced by *NFE2L2*-mediated gene expression profile seems to be more complicated because a number of genes involved in cell proliferation are transcriptionally modulated by *NFE2L2* through glutathione as the effectors.[16] The association of *NFE2L2* mutation and poor prognosis of lung cancers suggested that a direct role of *NFE2L2* mutation in tumor progression. Constitutive expression of *NFE2L2* could provide a survival advantage to invasive and metastatic cancer cells, by adaptation to microenvironment on evolution of chemoresistance in cancer cells under hypoxia.[17,18] Both the degree of CDDP-induced DNA crosslinking and the degree of number of apoptosed cells were increased significantly in A549 cells transfected with *NFE2L2*-siRNA.[19] The expression of multidrug resistance-associated proteins, the drug efflux proteins, was also significantly reduced in *NFE2L2*-silenced A549 cells.[19] A recent report showed that inhibition of *NFE2L2* function restored cisplatin (CDDP) sensitivity in human ovarian cancer SKOV-3 cells.[20]

Cross-talk between tumor hypoxia and induction of *NFE2L2* has also been suggested.[21] *NFE2L2* knockdown by *NFE2L2*-siRNA inhibited the proliferation of lung cancer cells.[19] The growth-inhibitory properties of *NFE2L2* suppression are likely to be attributable to its induction of cell cycle arrest at G1 phase in A549 and HCI-H292 cells.[19] The cell cycle arrest was associated with the repression of pRb phosphorylation in these cells.[19] Hypoxia stimulates the *NFE2L2* transcriptional response via EGFR kinase signaling.[22] EGFR-activated signaling and actin remodeling regulates cyclic-stretch-induced *NFE2L2* transcriptional response and subsequent antioxidant response element expression.[23] In our analysis, *EGFR* and *NEF2L2* were found to be exclusive with each other.

In vitro, wild type NFE2L2 was efficiently polyubiquitinated while mutant NFE2L2 proteins were only weakly polyubiquitinated after treatment with MG132.[10] Wild-type NFE2L2 protein decreased rapidly whereas mutant NFE2L2 proteins were degraded more slowly, having half-lives of approximately twice that of wild type.[10] In addition, mutant

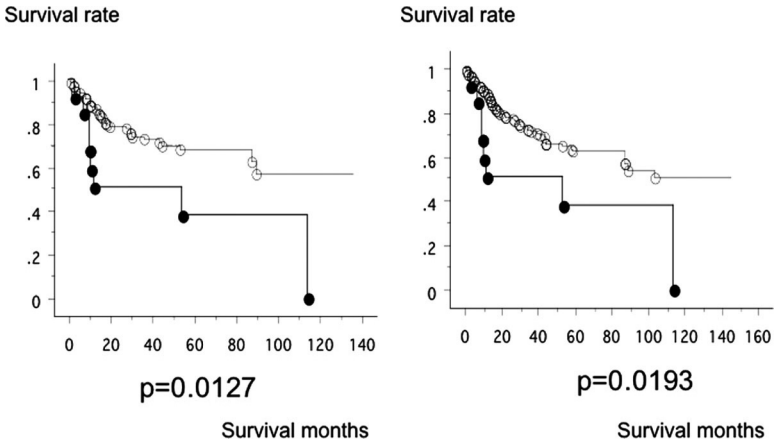


**FIGURE 3.** Kaplan-Meier curve of overall survival within squamous cell carcinoma (left) and within male (right) separated by *NFE2L2* mutation.

NFE2L2 proteins were significantly more active than wild type NFE2L2 by analyzing luciferase activity.

In summary, *NFE2L2* mutation may play a role in the progression of squamous cell carcinoma in male smokers. Hence, inactivation of the NFE2L2 pathway may become a therapeutic strategy to reinforce this treatment for this malignancy.

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